Gadolinium deposition in the brain in a large animal model: comparison of linear and macrocyclic gadolinium-based contrast agents

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Abstract: Objective: Recent studies reported a signal intensity increase in the deep cerebellar nuclei (DCN) on magnetic resonance images caused by gadolinium deposition after the injection of gadolinium-based contrast agents (GBCAs). There is an ongoing debate if the propensity of a GBCAto deposit gadolinium is primarily determined by its class as either linear or macrocyclic. In the current study, we aimed to compare the amount and the distribution of retained gadolinium of linear and macrocyclic GBCAs in the DCN after a single injection at a dose comparable to a human patient's in a large animal model. Materials and Methods: Eighteen sheep were randomly assigned in 6 groups of 3 animals, which received a single injection of 0.1 mmol/kg body weight of either the macrocyclic GBCAs gadobutrol, gadoteridol, or gadoterate meglumine; the linear GBCAs gadobenate dimeglumine or gadodiamide; or saline. Animals were euthanized 10 weeks after injection. Local distribution and concentration of gadolinium and colocalization to other metals (iron, zinc, copper) in the DCN was assessed by laser ablation-inductively coupled plasma-mass spectrometry. Results: Average gadolinium concentration for the macrocyclic GBCAs and the saline groupwas below the limit of quantification (5.7 ng/g tissue). In contrast, 14 (for gadobenate) and 27 (for gadodiamide) times more gadolinium than the limit of quantification was found for the linear GBCAs gadobenate (mean, 83 ng/g) or gadodiamide (mean, 155 ng/g brain tissue). Gadolinium distribution colocalized with other metals for linear GBCAs and a specific accumulation in the DCN was found. Discussion: The current study supports the hypothesis that the amount of gadolinium deposited in the brain is primarily determined by its class as either macrocyclic or linear. The accumulation of gadolinium in the DCN for linear GBCAs explains the hyperintensities in the DCN found in previous patient studies with linear GBCAs.

DOI: https://doi.org/10.1097/rli.0000000000000575

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Comparison of Linear and Macrocyclic Gadolinium-Based Contrast Agents

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Discussion: The current study supports the hypothesis that the amount of gadolinium deposited in the brain is primarily determined by its class as either macrocyclic or linear. The accumulation of gadolinium in the DCN for linear GBCAs explains the hyperintensities in the DCN found in previous patient studies with linear GBCAs.

Key Words: GBCA, gadolinium-based contrast agents, macrocyclic GBCAs, linear GBCAs

(*Invest Radiol* 2019;54: 531–536)

In the last 4 years, the debate on the deposition of gadolinium in patient's brains after serial injections of gadolinium based contrast agents (GBCAs) has been among the top issues debated in radiological science.[1] The debate started at the beginning of 2014 when Tomonori Kanda reported for the first time increasing hyperintensities on nonenhanced T1-weighted magnetic resonance imaging (MRI) scans in the dentate nucleus (DN) and the globus pallidus that correlated with the number of previous GBCA injections.[2] Further research showed that gadolinium stored in the brain tissue was the source of the hyperintensities and that also other parts of the brain were affected with the DN showing the highest amounts of gadolinium.[3–5] Subsequent studies reported that hyperintensities in the DN were exclusively found after the injection of linear GBCAs, but not after injection of macrocyclic GBCAs.[6,7] Ever since, a multitude of preclinical and clinical studies has been published assessing the propensity of the marketed GBCAs to cause hyperintensities or gadolinium deposition in the brain or other parts of the body.[1,5–18] The intensive scientific debate on gadolinium depositions culminated in 2017 with divergent regulatory actions. Although the European Union decided to remove all linear GBCAs (with a few minor exceptions) from the market using a "precautionary approach," the Food and Drug Administration issued a class warning for all marketed GBCAs.

The different propensity of linear and macrocyclic GBCAs to cause hyperintensities has been debated in more than 50 research articles in the last 4 years.[1,6–17] Although the majority of articles reported no signal intensity increase after macrocyclic GBCA injections, some positive findings[19] kept the debate alive if macrocyclic GBCAs can principally cause gadolinium accumulation in the DCN that finally becomes visible on MRI scans.

In the current study, we aimed to assess the concentration of gadolinium retained in the DCN in a large animal model 10 weeks after a single injection of the 3 marketed macrocyclic GBCAs gadobutrol, gadoterate meglumine, and gadoteridol; the nonionic linear GBCA gadodiamide; and the ionic linear GBCA gadobenate. Moreover, we used laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) to investigate if an accumulation of gadolinium can be identified in the DCN, which could explain the observed signal intensity increase that was reported on nonenhanced T1-weighted MRI scans.

MATERIALS AND METHODS

The study was conducted as an "add-on" study to an already ongoing study on teeth abrasion of sheep depending on the provided food. The study design followed the 3R requirements (replace, reduce, refine) and was in accordance with the ethical obligation to maximize knowledge that can be obtained from animal experiments. The study was conducted according to Swiss animal welfare act and approved by the local governmental authorities with the animal license number ZH235/17. Eighteen female Swiss-Alpine sheep with an age between 4 and 10 years and with a body weight of 80.8 ± 19.6 kg were randomly assigned (independent of age and body weight) to 1 of 6 groups with 3 animals each. The 5 treatment groups received an injection of one of the following GBCAs at 0.1 mmol/kg (standard dose, based upon body surface area normalization): Omniscan (gadodiamide, Omniscan; GE Healthcare AG, Wädenswil, Switzerland), Multihance (gadobenate-dimeglumine, MultiHance; Bracco Imaging Deutschland GmbH,

Received for publication January 26, 2019; and accepted for publication, after revision, March 27, 2019.

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The study was not supported by any funding.

Alexander Radbruch lectures for Guerbet and Bayer, and he is also part of the Advisory Boards for GE, Bracco, and Guerbet. This study was supported by Bayer and Guerbet. For the remaining authors, no conflicts of interest are declared.

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ISSN: 0020-9996/19/5409–0531
DOI: 10.1097/RLI.0000000000000575

Konstanz, Germany), Gadovist (gadobutrol, Gadovist; Bayer Vital GmbH, Leverkusen, Germany), Prohance (gadoteridol, ProHance; Bracco Imaging Deutschland GmbH, Konstanz, Germany), and Dotarem (gadoterate meglumine, Dotarem; Guerbet AG, Paris, France). The control group was injected with the equal volume of saline (0.2 mL/kg NaCl 0.9%; B. Braun Medical AG; Sempach, Switzerland).

The person performing the injections was blinded to the treatment groups as the syringes were randomly prepared by 2 persons unrelated to the study. All injections were done under manual restraint at the vena jugularis externa using an intravenous cathether (Surflo ETFE IV Catheter 18 gague × 2″ [Green], reference number SR-OX1851CA; Terumo Medical Corporate Office, New Jersey), which was removed directly after the injection. No clinical abnormalities were detected in the study cohort until termination of the experiment. The scientists who conducted the LA-ICP-MS and the gadolinium quantification were blinded to the GBCAs at all parts of the study.

Euthanization, Tissue Harvesting, and Sample Preparation

Ten weeks after the initial injections, the animals were anesthetized with 0.5 mg/kg body weight xylazine (xylazinum, Xylazin Streuli ad us vet; Streuli Pharma AG, Uznach, Switzerland) and 5 mg/kg body weight ketamine (Ketanarkon 100 ad us vet; Streuli Pharma AG) intramuscularly and euthanized with 100 to 150 mg/kg body weight pentobarbital (pentobarbitalum natricum, Esconarkon ad us vet; Streuli Pharma AG; 100–150 mg/kg body weight) intravenously. The death of the animals was confirmed by the reversal of the heartbeat and the pupillary reflex, and all tissues were harvested immediately thereafter. The brain was excised in total and divided into 2 hemispheres (Fig. 1). The left brain hemisphere was coronally cut into 1 cm slices and cryopreserved (at −80°C). Deep cerebellar nuclei of the left cerebellum were cut into a 50-µm-thick section and fixed on a piece of cork with Tissue-Tek O.C.T. Compound (Sakura Finetek GmbH, Staufen, Germany). For chemical analysis, deep cerebellar nuclei of each sheep were cut in thin sections of 10-µm thickness with a cryotome and mounted on microscopic glass slides. Before the ablation process, microscopic images were recorded with a BZ-9000 inverted fluorescence/bright field microscope (Keyence, Osaka, Japan). The right hemisphere of 18 sheep was formalin fixed (4% buffered formalin), paraffin embedded, and stored until further analysis. Much care was taken to avoid cross-contamination between samples, and all instruments were thoroughly rinsed before the next samples were processed.

Laser Ablation ICP-MS

Matrix-Matched Standards

Spatially resolved determination of gadolinium, copper, iron, and zinc within the cerebellum thin sections was performed via an external calibration based on matrix-matched gelatin standards. For the preparation of aqueous stock solutions, a gadolinium ICP-MS standard (1000 mg·L^{-1}; Merck KGaA, Darmstadt, Germany) was diluted. The calibration included 7 concentrations ranging from 0.05 µg·g^{-1} to 10 µg·g^{-1}. To ensure a good homogeneity, the mixture of gelatin (10%) and aqueous standard solutions were heated to 45°C and repeatedly mixed. The gelatin standards were sectioned with a cryotome (CryoStar NX70; Thermo Fisher Scientific, Bremen, Germany) to a thickness of 10 µm to match the thickness of the tissue samples and ablated using the same conditions for laser ablation and mass spectrometry immediately before the analysis of the respective cerebellum samples to ensure the comparability of the data. For each calibration standard, 11 lines were recorded over 30 seconds with a laser spot size of 40 µm and a scan speed of 120 µm·s^{-1}. The first line of each calibration standard was not included for data analysis due to a larger ablated area compared with the following 10 lines because of the ablation of small areas outside the adjusted laser spot size caused by heating processes. Validation of the matrix-matched standard concentrations was performed by bulk analysis. Gelatin standards (50 mg) were digested by adding 290-µL concentrated HNO_3 and diluted to a final volume of 10 mL. Further dilution resulted in a concentration range from 0.25 ng·g^{-1} to 10 ng·g^{-1}. Rhodium (ICP standard, 1000 mg·L^{-1}, VHG Labs, Manchester) as internal standard was added before digestion with a final concentration of 1 ng·g^{-1}. For external calibration, 6 Gd concentrations in the range from 0.1 ng·g^{-1} to 20 ng·g^{-1} with 1 ng·g^{-1} rhodium were prepared. The resulting concentrations after bulk analysis and the respective intensities monitored by ICP-MS were applied for linear regression.

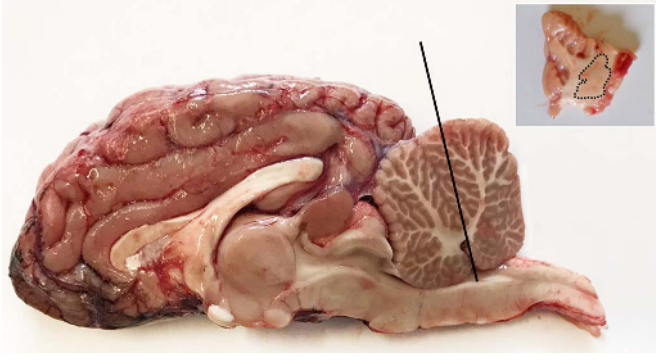

FIGURE 1. Photographic view on the medial side of the right hemisphere of a sheep brain during dissection.

Laser Ablation and ICP-MS Parameters

Laser ablation experiments were carried out using a laser ablation system model LSX 213 G2^{+} with a HelEX Active 2-Volume Cell (Teledyne CETAC Technologies) and a laser wavelength of 213 nm coupled via Tygon tubing to a triple quadrupole-based iCAP TQ ICP-MS (Thermo Fisher Scientific). A laser spot size of 40 µm and a scan speed of 120 µm·s^{-1} were selected in a line-by-line scan with a distance between the ablated lines of 0 µm to achieve a reasonable analysis time. To ensure quantitative ablation of the material, the laser energy was optimized using a laser frequency of 20 Hz. The laser-generated aerosol was transported into the ICP-MS by a carrier gas mixture of helium (800 mL·min^{-1}) and argon (900 mL·min^{-1}) added via a Y-piece directly after the ablation cell. To convert the transient signal recorded by the ICP-MS instrument during laser ablation analysis into a 2-dimensional image, an in-house developed imaging software (MassImager written by R.S., Research Group Karst, Institute of Inorganic and Analytical Chemistry, University of Münster) was used. The sample introduction system used for LA-ICP-MS consisted of a quartz injector pipe with an inner diameter of 3.5 mm. The triple quadrupole-based ICP-MS instrument was used with oxygen (0.3 mL·min^{-1}) as reaction gas in TQ mode, and gadolinium and phosphorus were monitored as oxygen reaction products (mass shift). Sampler and skimmer cones of the ICP interface consisted of nickel. The following MS conditions were used: 1550 W rf power, 14 L min^{-1} cooling gas flow, 0.9 L·min^{-1} nebulizer gas flow, and 0.8 L·min^{-1} auxiliary gas flow. The isotopes ^{158}Gd + ^{16}O and ^{160}Gd + ^{16}O were monitored with dwell times of 0.08 second each to achieve optimum detection limits based on a study by Clases et al[20] and Fingerhut et al,[21,22] whereas ^{31}P + ^{16}O, ^{57}Fe, ^{65}Cu, and ^{66}Zn were monitored with dwell times of 0.04 second each. For bulk analysis of the gadolinium concentration in the gelatin standards, the same ICP-MS instrument as for laser ablation analysis was used with standard liquid sample introduction fed by an ASX-560 autosampler (Teledyne CETAC Technologies). All analyses were carried out in KED (He as collision gas, 4.2 mL·min^{-1}) mode with the following ICP-MS conditions: 1550 W rf power, 14 L·min^{-1} cooling gas flow, 1.1 L·min^{-1} nebulizer gas flow, and 0.8 L·min^{-1} auxiliary gas flow. The isotopes ^{158}Gd, ^{160}Gd, and ^{103}Rh were monitored with dwell times of 0.1 second each.

Spatially resolved quantification of gadolinium in the DCN samples was conducted within thin sections. Because the DCN were not visible in the microscopic images, the complete thin section had to be investigated by means of LA-ICP-MS. Therefore, obtaining a reasonable compromise between analysis time, spatial resolution, and limit of detection for gadolinium was the main goal of the method development. A laser spot size of 40 μm and a scan speed of 120 $\mu m \cdot s^{-1}$ turned out to be most suitable for the detection of gadolinium, copper, iron, and zinc within a limited analysis time, nevertheless allowing the discrimination of the DCN from other brain structures. For calibration of gadolinium, matrix-matched standards based on gelatin were analyzed as well. Applying these settings, the limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the 3σ and 10σ criterion, respectively. In TQ mode of the ICP-MS instrument with oxygen as reaction gas, a limit of quantification for $^{158}Gd + {}^{16}O$ of LOQ = 5.7 $ng \cdot g^{-1}$ could be achieved (LOD = 1.7 $ng \cdot g^{-1}$). Finally, it was assessed by a histopathologist if the DCN could be identified on the copper, zinc, iron, and gadolinium LA-ICP-MS maps.

For evaluation of the mean gadolinium concentration in the DCN and the surrounding tissue, an area of the DCN—identified by increased iron thresholds in this region—was selected. Three regions of interest including 500 data points each (approximately 880 × 880 μm) within the threshold area were selected for averaging in each thin section within the DCN. Equally, 3 regions of interest outside the DCN (iron threshold region) were selected for averaging the gadolinium concentration in the surrounding tissue.

RESULTS

Visual Assessment of LA-ICP-MS Analysis

A clear colocalization of gadolinium and iron, copper, and zinc was found in all animals injected with linear GBCAs.

The LA-ICP-MS analysis enabled a clear identification of the 3 DCN for all 18 sheep when iron, copper, and zinc were analyzed. In contrast, when gadolinium was analyzed, the DCN could exclusively be identified in those 6 sheep that were injected with linear GBCAs. A clear colocalization could be found for gadolinium and iron, zinc, and copper in the LA-ICP-MS when linear GBCAs were injected. The results after LA-ICP-MS analysis for one representative sample of each group are shown in Figure 2. The qualitative distribution maps of iron, copper, and zinc, and the quantitative gadolinium distribution map are shown in Figure 3, B, C, D, and A, respectively.

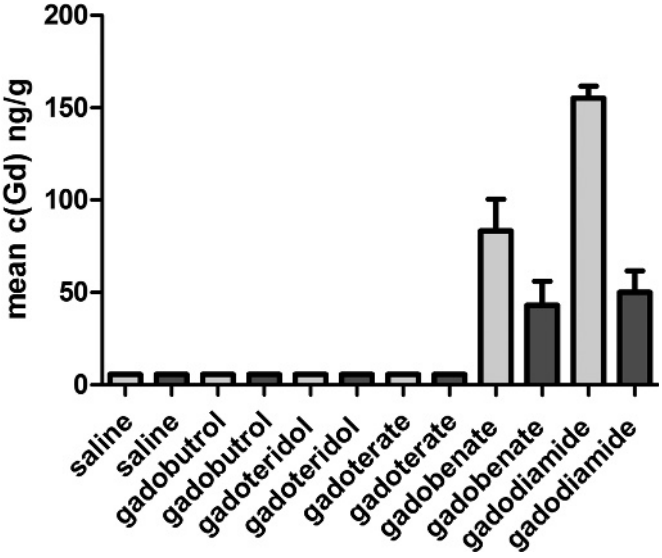

FIGURE 2. Gadolinium concentration in the DCN (light gray) and in the surrounding area (dark gray) for all GBCAs. The detection limit of gadolinium was not reached for any of the macrocyclic GBCAs.

Quantitative Analysis

For all animals injected with macrocyclic GBCAs or saline, the Gd concentration found in either the DCN or the surrounding tissue was below the limit of quantification (5.7 ng gadolinium/g tissue). In contrast, mean gadolinium concentrations above the limit of quantification between 58 ng gadolinium/g tissue and 167 ng gadolinium/g tissue were detected for the 6 animals, which were injected with the linear GBCAs gadodiamide or gadobenate.

Notably, the DCN of all 3 animals who received the linear nonionic GBCA gadodiamide showed a higher concentration of gadolinium (145, 154, 167 $ng \cdot g^{-1}$) than those of the animals who received theionic linear GBCA gadobenate (58, 76, 116 $ng \cdot g^{-1}$). Also, and in agreement with the visual assessment of the DCN, gadolinium levels for all animals that were injected with linear GBCAs were higher in the DCN than in the surrounding area of the DCN. The results for all analyzed animals are summarized in Table 1 and visualized in Figure 2.

DISCUSSION

In the current study, we found on average 14 times more gadolinium for gadobenate dimeglumine and 27 times more gadolinium for gadodiamide in the DCN of sheep than the limit of quantification, whereas no gadolinium at or above the limit of quantification was found for the saline group and for all 3 macrocyclic GBCAs. Moreover, we found a clearly visible accumulation of gadolinium in the DCN as well as a colocalization of gadolinium with zinc, copper, and iron in the group of animals that received linear GBCAs, whereas no accumulation of gadolinium was found when macrocyclic GBCAs were injected.

Our results are in accordance with the majority of published rodent studies[23–26] and provide further evidence that the dichotomization of GBCAs as either linear or macrocyclic is justified. In vitro experiments showed no gadolinium release for macrocyclic GBCAs 14 days after incubation of human serum at 37 degrees, whereas a gadolinium release of 20% was shown for the nonionic linear GBCAs and a release of 2% for the linear ionic GBCAs.[27] Based on this in vitro study[27] and animal studies,[23–25,28,29] the hypothesis has been developed that macrocyclic GBCAs remain in their chelated form and are washed out from the brain over time through the glymphatic system, whereas linear GBCAs partly dechelate in the brain in metal-rich areas by transmetalation.[18,30] A recent study by Deike-Hofmann[31] showed that an SI increase can be found first in the choroid plexus and subsequently in the CSF, suggesting a pathway of the intravenously injected GBCAs through the glymphatic system of the brain. It can be hypothesized that the "dechelation rate" of GBCAs on this pathway through the glymphatic system is determined by (1) the stability of the injected GBCA, (2) the amount of the injected GBCA, (3) the duration of the injected GBCA within the glymphatic system, and (4) the concentration and availability of potential transmetalation partners.

The colocalization of gadolinium in the DCN with zinc, iron, and copper that was found in the current study might be explained by the increased transmetalation of the GBCAs caused by an increased supply of potential transmetalation partners in the metal-rich area of the DCN. The theory of a partial transmetalation of linear GBCAs is in the current study also supported by the decreased amount of gadolinium found in the surrounding area that contain less metal compared with the DCN (Table 1, Fig. 2). The absence of any statistically significant gadolinium accumulation in the DCN for macrocyclic GBCAs is, moreover, in agreement with the theory that macrocyclic GBCAs do not dechelate in vivo to a significant degree and are excreted over time through the glymphatic system.[23,24]

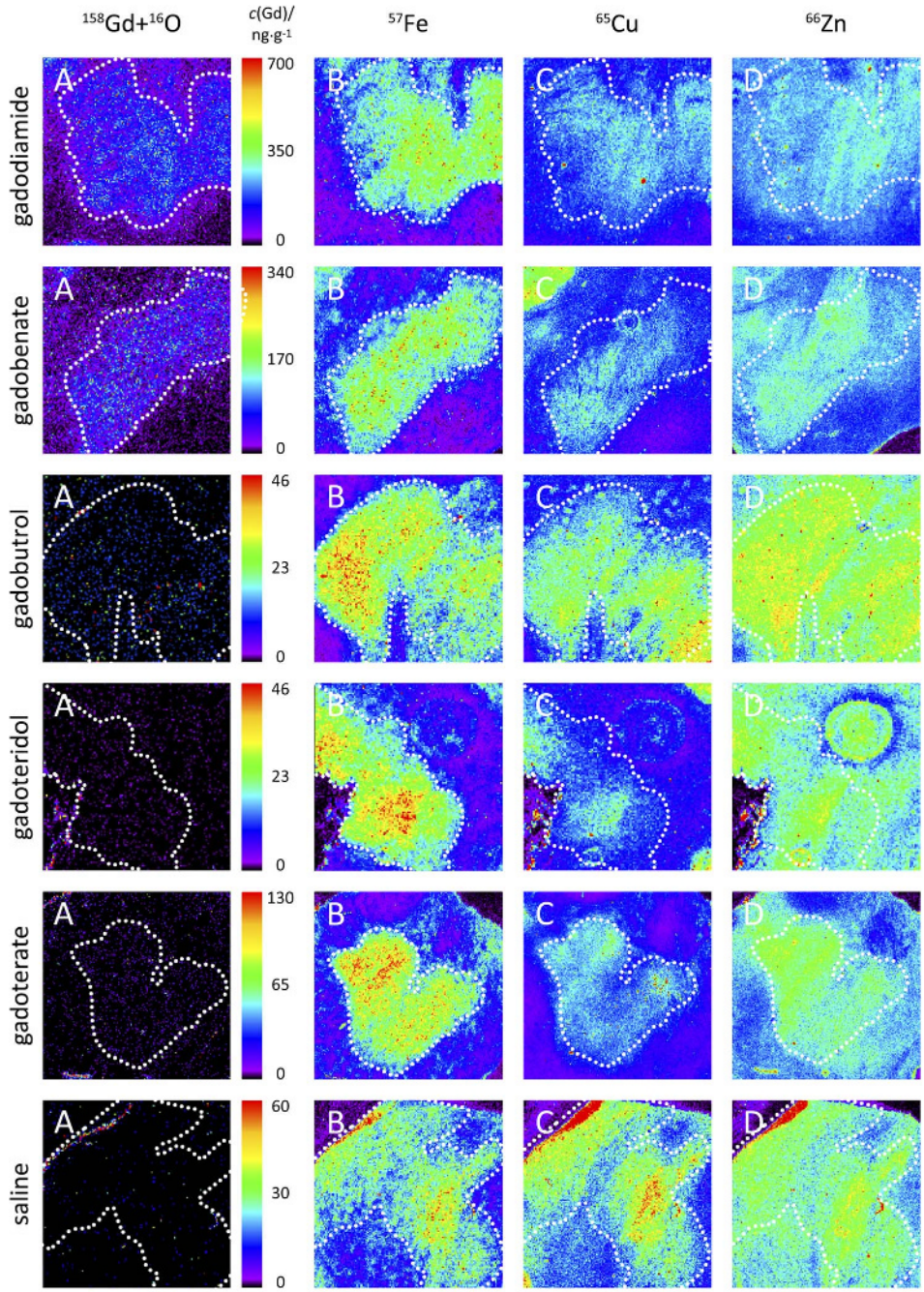

FIGURE 3. Representative results for the LA-ICP-MS analyses of cryo cerebellum samples of sheep treated either with gadodiamide, gadobenate, gadobutrol, gadoteridol, gadoterate, or saline solution. The quantitative distribution map of gadolinium ($^{158}Gd + {}^{16}O$) for each sample is shown in (A), and the qualitative distribution maps of iron (^{57}Fe), copper (^{65}Cu), and zinc (^{66}Zn) are depicted in (B, C, D). LA-ICP-MS analyses were performed with a laser spot size of 40 µm and a limit of quantification at 5.7 ng/g (LOQ). The deep cerebellar nuclei are marked by dotted lines. A clear colocalization of gadolinium displays for iron, copper, and zinc in all animals. In contrast, an accumulation for gadolinium could only be found for the linear GBCAs gadodiamide and gadobenate, whereas no accumulation was found for the macrocyclic GBCAs.

It is important to note that the excretion process of GBCAs through the glymphatic system requires time—an almost total washout was found in rodents 5 months after injection of a very high amount of macrocyclic GBCAs in the study by Robert et al.[24] Hence, studies that determine the total gadolinium content at a time point when the washout process is still in progress will result in a mixture of the intact gadolinium chelate that is in the washout process and the potentially long-standing gadolinium deposition of released gadolinium that is bound to different partners.[24,26]

The different duration between gadolinium injection and the animal euthanization is most likely also the reason for varying results of a

TABLE 1. Mean Gadolinium Concentration Within the DCN and in the Surrounding After Spatially Resolved Quantification Via LA-ICP-MS

	Mean c(Gd)/ng·g^{-1}		GBCA
Sheep Number	Within DCN	Surrounding of DCN	Administration
1	<LOQ, 3	<LOQ, 2	Gadobutrol
6	<LOQ, 3	<LOQ, 2	Gadobutrol
4	<LOQ, 2	<LOQ, 2	Gadobutrol
3	<LOQ, 4	<LOQ, 3	Gadoteridol
7	<LOQ, 3	<LOQ, 2	Gadoteridol
5	<LOQ, 4	<LOQ, 2	Gadoteridol
9	<LOQ, 2	<LOQ, 2	Gadoterate
11	<LOQ, 2	<LOQ, 2	Gadoterate
12	<LOQ, 2	<LOQ, 2	Gadoterate
8	116	69	Gadobenate
15	76	32	Gadobenate
2	58	28	Gadobenate
13	167	73	Gadodiamide
14	145	43	Gadodiamide
18	154	34	Gadodiamide
10	<LOD (1)	<LOD (1)	Saline solution
16	<LOQ, 3	<LOQ, 2	Saline solution
17	<LOQ, 2	<LOQ, 3	Saline solution

LOD = 1.7 ng·g^{-1}; LOQ = 5.7 ng·g^{-1}.

DCN indicates deep cerebellar nuclei; LA-ICP-MS, laser ablation-inductively coupled plasma-mass spectrometry; GBCA, gadolinium-based contrast agent; LOQ, limit of quantification; LOD, limit of detection.

rodent study by McDonald et al[32] that showed only moderate differences between gadolinium retention of linear GBCAs (gadodiamide: median, 6.9; gadobenate: median, 4.7 μg/g of brain tissue) and the macrocyclic GBCA gadobutrol (median, 1.6 μg/g of brain tissue). In the McDonald study, the animals were euthanized 9 days after injection, which might not be a sufficient period to allow a clearance of the macrocyclic GBCA gadobutrolfrom the glymphatic system. Notably, no gadolinium was found 9 days after injection in the McDonald study for the macrocyclic GBCA gadoteridol, which might—at least for the analyzed rodent model—support the hypothesis of different washout kinetics for macrocyclic GBCAs. Finally, it needs to be noted that McDonald et al injected 50 times higher gadolinium dosages (20 injections of 2.5 mmol/kg) than the current study (single injection of 0.1 mmol/kg), which might have influenced the washout process.

Generally, gadolinium in any chenical form (with higher relaxivities if bound to macromolecules) first becomes visible in MRI if a threshold of approximately 1 μg/g of tissue is exceeded. Hence, even for the animals injected with linear GBCAs (highest amount for gadodiamide: average, 155.33 ng/kg tissue), we would have most likely not been able to show any signal intensity changes in MRI. For humans, it has been shown that hyperintensities in the DN finally become visible after 6 injections of linear GBCAs (0.1 mmol/kg body weight linear).[6,12] Interestingly, the level of detection for the visibility of hyperintensities in the DN of 1 μg would have been reached after approximately 6 injections of 0.1 mmol in the current sheep model (6 times 155.53 ng ~ 1 μg), which might support the comparability of the sheep model and the conditions in human beings. Importantly, the results of our study can explain the fact that a signal intensity increase on T1-weighted MRI scans could be found in the majority of the published studies when linear GBCAs were injected,[2,11,12,14–16,33] whereas no effect was found in the majority of studies that investigated macrocyclic GBCAs.[6,8–10,13]

Because we did not find any gadolinium accumulation in the DCN for macrocyclic GBCAs, it is, from a methodological point of view, impossible that any SI increase on T1-weighted MRI scans is caused by macrocyclic GBCAs in the current experimental setup. However, it cannot be excluded that gadolinium accumulation in the DCN appears finally when higher dosages of macrocyclic GBCAs are applied. Considering the strong differences of gadolinium deposition between linear and macrocyclic GBCAs found in the current study, and the fact that hyperintensities after serial administrations of linear GBCAs also first become apparent after approximately 6 injections,[11] it seems highly unlikely that the administration of macrocyclic GBCAs might ever reach the level that a visible signal intensity increase in the DCN can be caused in a clinical setting.

Our study is different to the majority of published animal studies assessing gadolinium retention in 2 aspects. First, we used a large animal model, whereas the previously published studies primarily focused on rodent models. Second, we investigate gadolinium retention after the injection of a single clinical dosage of GBCAs—with the same concentration (0.1 mmol/kg) that is used in patients in clinical practice.

The reason for using a sheep model instead of the established rodent model is that the sheep anatomy is much more similar to the human anatomy and it has recently been correctly emphasized that the rodent model might not be optimal for the investigation of gadolinium deposition.[34] Moreover, this approach enabled us to assess the gadolinium deposition exclusively in the DCN because this area is far larger in sheep compared with rodents, and a precise delineation of the DCN could be guaranteed. In contrast, the previously published studies on rodent brains determined the total concentration of gadolinium in the cerebellum, which might have included gadolinium in the glymphatic system during the washout process and hence not adequately reflect gadolinium in the cerebellar nuclei.

We assessed gadolinium deposition after a single injection of GBCAs in the current study to simulate the clinical conditions under which the majority of GBCA injections are performed in clinical practice. The study was conducted in a prospective approach as an add-on study to an ongoing study that aimed to determine the influence of the food composition on tooth abrasion. Therefore, the study design was restricted, and the ability to increase the applied amount of GBCAs was limited. Although the study design followed the 3R requirements (replace, reduce, refine) and was in accordance with the ethical obligation to maximize knowledge that can be obtained from animal experiments, it did not allow us to prolong the time between injection and euthanization of the sheep. Generally, it is favorable to euthanize the animals a long period after the last injection of GBCAs to determine long-term gadolinium deposition. The selected period of 10 weeks was the longest interval that could be achieved under the conditions of the ongoing study. However, the fact that no significant difference was found between the gadolinium amount retained for macrocyclic GBCAs and the control group supports the hypothesis that the washout process of macrocyclic GBCAs from the glymphatic system was almost completed.[31]

Limitations of our study must be acknowledged. First of all, the low amount of injected gadolinium unfortunately impeded a speciation analysis of the retained gadolinium.

Moreover, an obvious limitation is the fact that all animals were euthanized after 10 weeks, and hence we cannot conclude if the found gadolinium for the linear GBCAs will remain in the brain or be excreted after a longer period. However, given the proposed pathomechanism of a partial dechelation and the pharmakokinetic modeling performed in the study by Robert et al[24] and Jost et al,[23] it seems likely that at least part of the gadolinium will be trapped resulting in long-term gadolinium deposition.

As mentioned previously, a further limitation of our study is that we only examined a single dose of GBCAs, and we hence cannot exclude that traces of gadolinium from linear or macrocyclic GBCAs can be found when higher dosages are applied. However, we wanted to focus

on the most relevant clinical situation, which is the injection of a single dosage of GBCAs. The results of our study might reassure patients that there is no measurable gadolinium in the DCN compared with the background level of gadolinium 10 weeks after injection of a single dose of macrocyclic GBCAs in a large animal model.

To summarize, the current study provides evidence from a large animal model that linear GBCAs leave traces of gadolinium within the DCN, while there was no significant difference of gadolinium concentrations between all 3 marketed macrocyclic GBCAs and the control group. Moreover, the specific accumulation of gadolinium from linear GBCAs as well as the colocalization with different metals in the DCN support the hypothesis that a partial dechelation of the less stable linear GBCAs is the cause of gadolinium deposition as well as of the signal intensity increase in the DCN on T1-weighted MRI scans caused by linear GBCAs.

ACKNOWLEDGMENT

The authors are grateful to Marcus Clauss and Jean-Michel Hatt for the opportunity to conduct a prospective add-on study based on their ongoing study, as well as Agnieszka Anna Karol, Adriano Wang, Gian-Luca Steger, and Sandra Bolliger for their great experimental support during blinding/randomization and tissue harvesting. In addition, the authors thank Urs Müller and Katrin Süss for their generously provided equipment, as well as Andrea Rothaus and Andrea Wagner for their expert technical assistance.

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